Functional Antibody Immobilization on 3-Dimensional Polymeric Surfaces Generated by Reactive Ion Etching

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Reactive ion etching (RIE) was used to pattern antibodies onto the surfaces of polymer substrates. A low pressure, inductively coupled oxygen plasma was used to anisotropically etch 25−30 μm deep features into poly(methyl methacrylate) (PMMA), Zeonex, and polycarbonate (PC). Scanning electron microscopy and contact angle measurements show that the resulting surfaces exhibit significant microroughness and enhanced hydrophilicity. Fourier transform infrared spectroscopy suggests that, in addition to enhanced surface area, chemical modifications may contribute to antibody immobilization. Polyclonal antibodies preferentially bind to the etched areas in RIE-patterned PMMA and Zeonex substrates but localize in unetched regions of RIE-patterned PC surfaces. Simple immunoassays were performed to demonstrate a potential application for RIE-modified polymer surfaces. Antibodies specific for the capture of fluorescently labeled cholera toxin, *S. aureus* enterotoxin B, and *B. anthracis* protective antigen were immobilized onto etched PMMA surfaces and shown to specifically capture their labeled antigen from solution. This work demonstrates a potentially useful fabrication methodology for constructing antibody microarrays on plastic substrates.

Introduction

A powerful tool within the field of proteomics is the protein microarray. An extension of DNA microarrays used in genetic analyses, protein microarrays offer the possibility of rapid and reliable profiling of the expression levels and interactions between proteins involved in signaling and disease pathways[1,2] or other events of biological significance.[3] Advances within the area of protein microarray technology have generally focused on the development of novel substrates for immobilizing functional proteins, the miniaturization of the size of functional features one can print and interrogate on a slide surface, and strategies for detecting specific protein−protein interactions while inhibiting nonspecific protein−protein or protein−substrate interactions.[4]

Proteins are commonly arrayed (printed) onto planar glass substrates that have been surface-derivatized with chemical functionalities such as epoxides or aldehydes to immobilize the protein through covalent bonds formed between nucleophiles on the protein and electrophiles on the slide surface. Detection of an interaction between the immobilized protein, commonly an antibody, and its partner in solution, e.g., capture of a fluorescently labeled antigen from solution, is usually achieved by spatially correlating the fluorescent signal with the known location of the printed capture reagent. Although printed protein (micro)arrays can be quickly and easily generated, successful implementation necessitates modified glass slides, a contact or piezoelectric dispenser capable of depositing small volumes, and robust handling protocols to ensure good signal-to-noise ratios.[5]

Polymeric supports may provide a suitable and versatile alternative to the use of chemically sophisticated glass substrates.[6] Although often unsuitable for short-wavelength fluorescence-based detection strategies due to intrinsic fluorescence or opaqueness, many polymers, such as Zeonex, poly(methyl methacrylate), and polycarbonate, do not suffer from these detractions and are suitable for use in longer-wavelength (λ > 520 nm) fluorescence-based detection schemes. Previous applications of polymeric supports for protein immobilization have relied on surface functionalization to enhance the protein capture and retention properties of the polymer, most notably by polymer grafting[7] or other modification to generate surface-localized carboxylic acid groups that can be activated and coupled to the amines of proteins for immobilization.[8,9] The enzyme-linked immunosorbent assay (ELISA), in which antibodies or antigens are chemisorbed onto polystyrene surfaces, is perhaps the most common application of polymeric supports in this regard.

An important trend in protein microarray development is the move from planar substrates to 3-dimensional supports.[10−12] Rather than immobilize only in the two dimensions available on a planar substrate, the addition

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of a third dimension yields superior signal over flat substrates because additional capture molecules can be immobilized within the depth of the support. A commercially available example of this approach employs nitrocellulose pads fixed onto the surface of a glass support. Due to their high immobilization efficiency and wetting properties, nitrocellulose pads can be printed on directly. Importantly, nitrocellulose pads enable enhanced signal output as a result of fluorescence from molecules immobilized not just on the surface but throughout the 3-dimensional matrix. In the area of purely polymeric supports, laser ablation, a technique that removes material from an exposed surface, has been employed to create textured, 3-dimensional surfaces that enhance the quantity of adsorbed protein per unit area,[13] resulting in greater fluorescent signal at the ablated areas as compared to the nonablated surface.[14]

We report the use of plasma-etched polymers as alternative substrate materials for protein arraying applications involving fluorescence-based detection. Reactive ion etching (RIE) using an inductively coupled oxygen plasma was applied to three different planar polymeric substrates, poly(methyl methacrylate) (PMMA), Zeonex and polycarbonate (PC), to generate textured, 3-dimensional surfaces for enhanced protein immobilization and signal optimization. In RIE, a plasma etches the surface of a substrate using both chemically reactive species and ion bombardment, and the resulting volatile byproducts are removed in vacuo during the process. Ion bombardment is necessary to achieve anisotropic etching but may induce physical damage to the substrate. Although both ion density (flux) and ion energy are regulated by a single radio frequency (RF) power source in conventional RIE, high-density plasma etching using an inductively coupled plasma (ICP−RIE) allows ion density and ion bombardment energy to be separately controlled in order to achieve high etch rates with minimal physical damage to the substrate. Previous applications of RIE in biology have focused on using the technique to generate textured polymeric surfaces for cell culture assays, presumably by affecting the interactions between proteins (or other biomolecules) on the cellular surface and the etched polymers.[15]

Furthermore, we report the application of RIE-treated polymers to perform fluorescence-based immunoassays without the need to chemically block the nonarrayed surface.[16] Protein−protein interactions detected fluorescently on planar substrates can suffer from an undesirably high background if nonspecific protein-surface binding is not sufficiently controlled by "blocking" the unused surface. Noise suppression is generally achieved with nonspecific biomolecules, such as bovine serum albumin, and elimination of a separate surface-blocking step could be useful in protein patterning and bio-detection applications.

In addition to the antibody patterning and immunoassay results reported here, the surface analysis techniques of scanning electron microscopy (SEM), water contact angle measurements, and Fourier transform infrared (FTIR) spectroscopy were employed to physically and chemically characterize the etched polymers in an effort to understand the cause of protein adsorption on the surface of RIE-etched polymers. We speculate that, in addition to the enhanced roughness and porosity of the etched surface, there may be chemical effects, as evinced by enhanced surface hydrophilicity, that contribute to the greater retention of adsorbed protein on the etched surface as compared to the native surface.

Experimental Section

Fabrication of RIE-Patterned Polymeric Substrates. Cell-cast Acrylite OP-1 PMMA sheets (1.0 mm thick) were purchased from Cyro Industries (Rockaway, NJ). Calibre 301−6 polycarbonate sheets (1.0 mm thick) were obtained from Dow Chemical Co. (Midland, MI). Injection molded Zeonex 480R plaques were obtained from Zeon Chemicals (Louisville, KY). Polymer slides (25 mm × 75 mm) were cut from these commercially available PMMA, PC, and Zeonex sheets. The substrates were cleaned with methanol using lint-free clean room wipes. All plasma etching was performed in an Oxford Plasmalab 100 ICP system (Oxford Instruments, Concord, MA). Approximately 25−30 μm deep, 1.0−1.5 mm diameter holes were etched in an array using a stainless steel stencil mask obtained from Kimball Physics (Wilton, NH). The RIE process parameters used were: O_2 = 100 sccm, ICP Power = 300 W, RF Power = 100 W (DC bias = 328 V) and temperature = −10 °C.

SEM. SEM images of the surfaces of 25−30 μm deep etched holes in RIE-patterned PMMA, Zeonex, and PC substrates were captured on a field emission scanning electron microscope, JEOL 6400F (JEOL-USA, Inc., Peabody, MA), with a 2−4 kV beam from graphite-coated samples.

Contact Angle Measurements. Contact angle measurements were conducted utilizing a Kruss (Kruss USA, Matthews, NC) G40 Contact Angle Measuring System. Doubly distilled water was used as the measured medium. Unetched polymer was cleaned with both acetone and 2-propanol rinses and dried with nitrogen before measurements were taken. Surface-etched polymer was not treated before measurements were taken. Droplets were manually introduced onto the surface with a microsyringe. Digital snapshots were taken of the droplets on the surface and analyzed with software provided by Kruss. Five replicates for each measurement were conducted to establish statistical significance.

FTIR. IR spectra of etched and unetched (control) polymeric substrates were collected on a Digilab FTS-7000 (Randolph, MA) using Harrick Scientific's Horizon, a horizontal attenuated total reflection (ATR) accessory (Ossining, NY) with a ZnSe ATR crystal. For each spectrum, 64 scans with 4 cm^{-1} resolution were averaged.

Safety Consideration. All work with toxins was performed at the Chemical and Radiation Detection Laboratory, a biosafety level 2 facility, at Sandia National Laboratories (Livermore, CA). Strict safety and security precautions were exercised in storage and handling of toxin samples. Safety procedures, as indicated in the appropriate MSDS forms, should be adhered to at all times. A 20% (v/v) bleach solution was used to disinfect all equipment, benchtops, and instruments. The disinfectant treatment was followed by a water rinse. Solutions containing toxins, as well as contaminated disposables, were treated with a 20% (v/v) bleach solution prior to disposal.

Chemicals. Polyclonal mouse α-goat and goat α-mouse antibodies labeled with the fluorophore Cy3 were purchased from Zymed, Inc. (South San Francisco, CA). Antibodies were supplied as a 1 mg/mL solution supplemented with 10 mg/mL BSA and purified away from the BSA additive using a Protein-G Nab kit from Pierce (Rockford, IL). Monoclonal α-cholera toxin (α-CT) and α-*S. aureus* enterotoxin B (α-SEB) antibodies were purchased from Biødesign (Saco, ME) as concentrated solutions and diluted as needed. Monoclonal α-*B. anthracis* protective antigen (α-PA) was from Advanced Immunochemicals (Long Beach, CA). Rhodamine labeled β-subunit of cholera toxin (CT*) and unlabeled diphtheria toxin (DT) were purchased from ListLabs, Inc. (Campbell, CA), *B. anthracis* protective antigen (PA) was from EMD Biosciences (San Diego, CA), and *S. aureus* enterotoxin B (SEB) was from Sigma-Aldrich (St. Louis, MO). PA, DT, and

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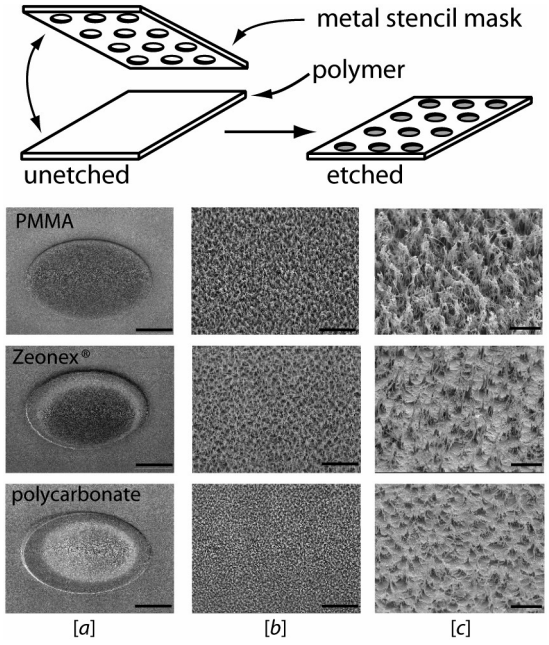

Figure 1. (top) Schematic of masking technique. Discrete, roughened 1 mm wide, 25−30 μm deep features are fabricated by applying a stencil mask to the surface of the polymer and allowing reactive ions to bombard the unmasked surface. The RIE process parameters used were: $O_2 = 100$ sccm, ICP Power = 300 W, RF Power = 100 W (DC bias = 328 V) and temperature = −10 °C. (bottom) SEM images of Zeonex, PMMA, and PC post-RIE. (a) Scale bar represents 500 μm. (b) Center of etched area; scale bar represents 50 μm. (c) Center of etched area; scale bar represents 10 μm.

SEB were labeled with AlexaFluor 532 from Molecular Probes (Eugene, OR) and purified as per the manufacturer's instructions. The fluorescently labeled species will be henceforth referred to as PA*, DT*, and SEB*. All immobilizations on polymeric surfaces and immunochemical assays were performed in phosphate buffered saline (PBS, pH 7.4) supplemented with 0.1% (v/v) of the detergent Tween 20 (PBST).

Antibody Patterning. For patterning experiments, a fluorescently labeled polyclonal antibody solution was applied in 50 μL volumes, sufficient to cover adjacent etched and unetched portions, and allowed to incubate for approximately 1 h. Postincubation, the surface was liberally washed with PBST. After washing away the buffer in Milli-Q water, the polymer was air-dried for imaging. Imaging was performed using the 532 nm excitation source of an Axon Instruments (Union City, CA) microarray scanner. A typical PMT gain setting was ≥700 V with laser power at 33%. Pixel size was set to 40 μm/pixel. Data from experiments were quantified by densitometry and a comparison of the raw fluorescence intensity on the RIE treated surface vs the native polymer surface provides a measure of the differential affinity of the antibody for the treated surface over the untreated surface.

Immunoassays. α-CT, α-SEB, or α-PA was applied at a concentration of 1 mg/mL over both the roughened and native surfaces in 50 μL aliquots and allowed to incubate for 1 h. Monoclonal antibody solution was removed and after a quick wash with PBST, antigens were applied in 50 μL aliquots and allowed to interact with the immobilized antibodies for 1 h. Excess antigen was removed, the surface was liberally washed with PBST, rinsed with Milli-Q water, air-dried, imaged, and quantified as described above.

Results and Discussion

Surface Characterization. To fabricate discrete, RIE-roughened features on the polymer, as shown in Figure 1, a metal stencil mask was laid over the polymer and the surface accessible through the open areas of the mask was selectively exposed to reactive oxygen plasma. SEM images (Figure 1) show that the 25−30 μm deep etched areas in RIE-patterned PMMA, Zeonex, and PC substrates exhibit significantly more microscopic roughness and, hence, have a more 3-dimensional topography than the untreated surface. These etched surfaces are characterized by grass-like protrusions and exhibit demonstrable roughness at various length scales (Figure 1, columns a, b, and c). The resulting surfaces are not all similar, and in particular the etched PMMA surface, with its high density of grass-like features, has a larger surface area than etched Zeonex and PC.

Water contact angle measurements, reported in Table 1, demonstrate that relative to untreated surfaces, oxygen RIE etched substrates exhibit dramatically enhanced wettability. Immediately after RIE treatment, Zeonex and PC surfaces exhibit no measurable contact angle, i.e., the water droplet wets out, indicating that these surfaces are superhydrophilic.[17,18] In contrast, the PMMA surface immediately after etching exhibits a contact angle of 30°, which decreases rapidly to 0° after 3 min. The droplet eventually wets out, transitioning from a higher energy state to a lower energetic configuration as it penetrates into the cavities of the porous PMMA network. SEM images (Figure 1) clearly show that the surface of etched PMMA is more textured than either Zeonex or PC, and this topographical difference may explain why delayed wetting is only observed for this polymer. Observed changes in water contact angle over time for etched PMMA, Zeonex, and PC indicate an aging effect, which can be attributed to polymer chain motion reorienting the polar groups into the bulk,[19] but it is noteworthy that after 20 days of exposure to air the etched areas still remain more hydrophilic than the unetched surfaces (Table 1).[15,20] Although oxygen RIE would be expected to generate polar functional groups that promote wetting, the induced roughening is also likely to contribute to the enhanced hydrophilicity, as suggested by mathematical models and experiments on roughened, hydrophilic surfaces.[17,21,22]

FTIR spectra provide evidence that chemically altered surfaces on etched PMMA and Zeonex may contribute to the observed protein immobilization on these areas (Figure 2, panels a and b, respectively). No spectral differences between etched and unetched PC were detected with this technique (data not shown), suggesting that RIE treatment does not chemically alter the PC surface in a manner that is detectable by FTIR. Oxygen plasma treatments applied to polymeric surfaces generally give rise to a number of hydrophilic oxidation products, such as terminal carboxyl,

Table 1. Contact Angles ± Standard Deviation for Distilled Water Droplets on Different Polymer Surfaces[a]

	PMMA	Zeonex	PC
unetched	85.3° (± 1.7°)	91.7° (± 0.6°)	85.3° (± 2.3°)
etched: immediately after RIE	30.5° (± 3.5°), then after 3 min droplet wets out	droplet wets out immediately	droplet wets out immediately
etched: 1 day after RIE	30.6° (± 3.5°), then after 3 min droplet wets out	droplet wets out immediately	droplet wets out immediately
etched: 20 days after RIE	29.0° (± 3.2°)	63.7° (± 0.1°)	31.4° (± 0.6°)

[a] Each value represents an average of 5 measurements.

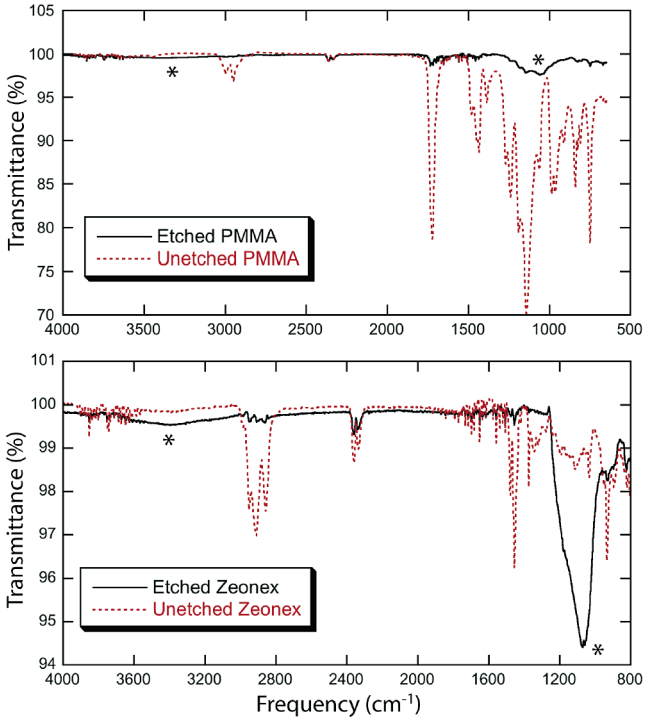

Figure 2. FTIR spectra of (a) PMMA and (b) Zeonex collected before and after RIE. The broad absorption feature in the range of 1050−1150 cm^{-1} likely indicates the presence of ether functionalities, and the broad absorption feature at 3200−3600 cm^{-1} indicates the presence of water adsorbed from the air. Asterisks indicate these bands.

carbonyl, or O-atom insertion products, such as ether functionalities.[19] No IR absorption at 1630−1780 cm^{-1} is observed for either etched PMMA or Zeonex, indicating undetectable levels of carbonyl-containing functionalities. A broad absorption feature in the range 1050−1150 cm^{-1} is detected on etched PMMA and Zeonex. Since contact angle measurements indicate that RIE dramatically increases surface hydrophilicity, which in turn suggests the incorporation of polar functional groups, this absorption may correspond to the carbon−oxygen single bond stretch of ether functionalities. Additionally, the broad IR absorption in the 3200−3600 cm^{-1} range indicates the presence of adsorbed water on the etched PMMA and Zeonex surfaces.

Antibody Immobilization on RIE-Patterned Polymeric Surfaces. RIE-patterned polymer surfaces were investigated for their protein immobilization properties. Previous observations in our lab showed that antibody solutions printed onto roughened surfaces resulted in deposited protein that was resistant to removal under washing conditions.[23] Using a stencil mask, circular features as small as 1.0 mm in diameter and 25−30 μm deep were etched into the surface of the native polymer (Figure 1). After a 1-h incubation with Cy3-labeled polyclonal antibodies and subsequent rinse, the fluorescence signal collected from the etched regions of the RIE-patterned substrates showed that the antibodies bound strongly to the etched surfaces (Figure 3) of PMMA and Zeonex. An approximately 20-fold larger fluorescence signal was collected within the boundary of the roughened area as compared to that outside the boundary (Figure 3a,b). Concentration dependence was examined for the patterning of goat α-mouse on PMMA. The fluorescence signal increased as the amount of applied antibody was increased up to 330 μg/mL (Figure 3d). For PC (Figure 3c), the single-concentration point pattern observed was opposite to that of PMMA or Zeonex, with protein localizing less to the RIE-etched area. We are currently investigating this phenomenon, but, as a preliminary explanation, we do note from FTIR analysis that in contrast to PC, the surface chemical structure of PMMA and Zeonex appears to be chemically altered by reactive oxygen plasma, suggesting that modifications, such as O-atom insertion

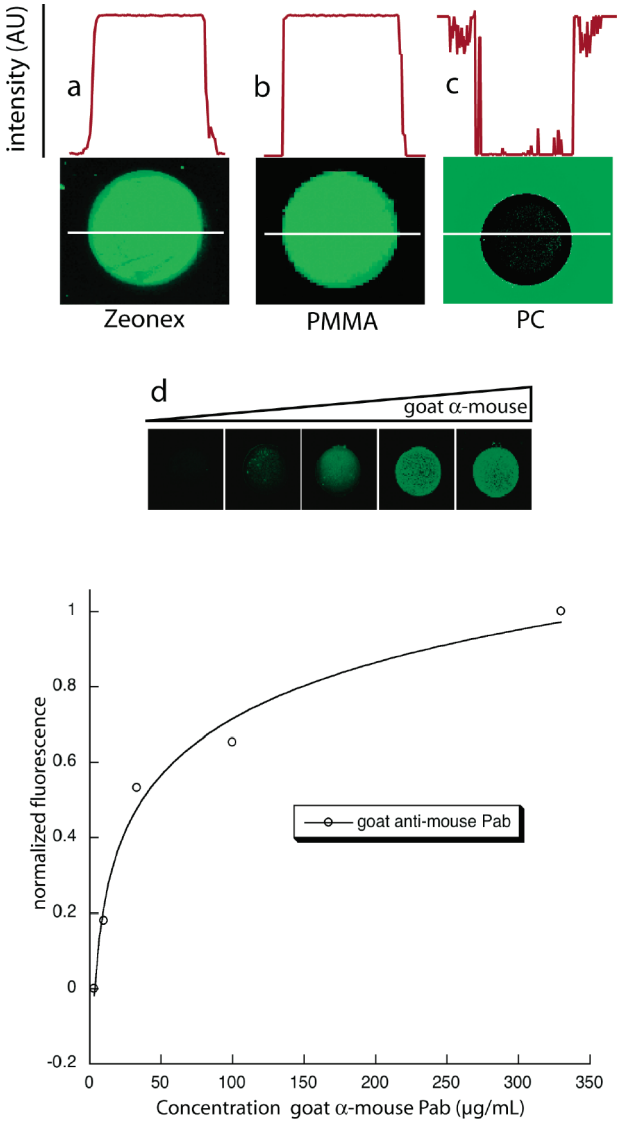

Figure 3. Antibody immobilization. (a, b, c) Fluorescence images of etched polymer substrates after Cy3-labeled polyclonal antibody (1 mg/mL in PBST buffer) incubation and washing steps. The white line across each image indicates the location where the fluorescence signal, displayed above each image, was measured. (d) Cy3-labeled goat α-mouse polyclonal antibody patterns in a concentration dependent fashion from 10 to 330 μg/mL of added antibody, as shown in image panel and accompanying intensity vs concentration graph. Data points correspond to normalized fluorescence signal obtained by integrating over each feature. The data were nonlinear least-squares fit to the log of the concentration.

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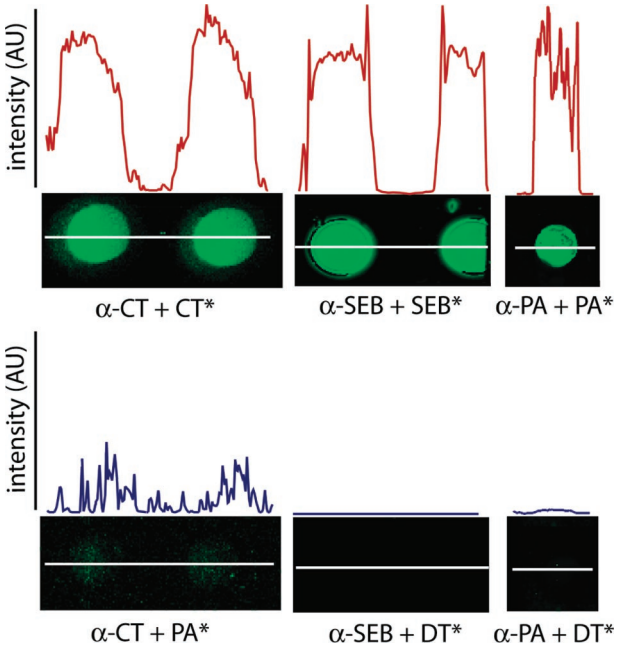

Figure 4. Immunoassays on etched PMMA. (top) Fluorescence images of etched PMMA substrates after a two-step immunoassay for CT*, SEB*, and PA*. (bottom) Companion control experiments performed on unetched PMMA substrates. Fluorescence intensity, as quantified along the white line, is represented graphically above each image.

to generate ethers, may be critical for protein uptake. This could imply that PC undergoes an etch mechanism in oxygen plasma that results in a less chemically altered surface than either PMMA and Zeonex. Indeed, while the dominant effect of polymer etching is usually chain scission, it has been demonstrated that a commercial-grade PC surface primarily undergoes cross-linking in high-density hydrogen, nitrogen and oxygen plasmas.[24]

Direct Immunoassays on RIE-Patterned Polymeric Surfaces. The selective patterning and immobilization properties described above were employed in a direct immunoassay whereby an immobilized antibody captures a fluorescently labeled antigen from solution. We employed monoclonal α-CT, α-SEB, or α-PA for the detection of CT*, SEB*, and PA*, respectively. The application of monoclonal antibody-containing solution to the polymer, in the absence of a chemical blocking step, followed by rinsing and application of 10 μg/mL of labeled cognate antigen demonstrated sufficient antibody immobilization for function in the immunoassay (Figure 4, top). When a cross-reactive, labeled protein, also at a concentration of 10 μg/mL, was added to test the specificity of the immobilized antibody (Figure 4, bottom), low fluorescent signal was observed, indicating the antibody retains binding specificity (low cross-reactivity).

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Conclusions

We have demonstrated that oxygen ICP−RIE modifies both the surface topography and surface chemistry of PMMA, Zeonex and PC. Etched polymer surfaces exhibit significantly enhanced microscopic roughness and increased hydrophilicity compared to unetched substrates. We initially proposed that RIE-roughened 3-dimensional polymer surfaces would enable enhanced loading and immobilization of proteins and proteinaceous recognition elements compared to planar native surfaces, and we found this to be the case for PMMA and Zeonex. In contrast, we found little protein adsorption in the etched regions of PC. FTIR analysis indicates that the surface of PMMA and Zeonex is chemically altered by RIE whereas no spectral differences were detected between etched and native PC. This implies that the incorporation of certain RIE-induced functional groups may be critical for protein loading. Future work will employ X-ray photoelectron spectroscopy (XPS) and secondary ion mass spectrometry (SIMS) to elucidate the chemistries of the RIE-treated surface that contribute to the selective polymer-antibody interactions that we have observed.

As a demonstration of a potential application of the RIE-fabrication method, monoclonal antibodies were directly applied to etched PMMA surfaces and assayed for their ability to capture fluorescently labeled antigen from solution. The patterned monoclonal antibodies were found to retain both their function and specificity for their unique antigen. Moreover, RIE-fabricated polymeric surfaces for protein immobilization may obviate the need for a blocking step to prevent nonspecific interactions from occurring outside the area containing the immobilized protein of interest. This initial work demonstrates a novel fabrication methodology for generating polymeric substrates useful for fluorescence-based immunoassays and protein patterning.

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